The EMBO Journal vol.5 no.12 pp.3235–3241, 1986

Sequence-specific DNA binding of the progesterone receptor to the uteroglobin gene: effects of hormone, antihormone and receptor phosphorylation

Alain Bailly, Claudine Le Page, Michel Rauch and Edwin Milgrom

Groupe de Recherche sur la Biochimie Endocrinienne et la Reproduction (INSERM U.135), Faculté de Médecine Paris-Sud, Le Kremlin-Bicêtre Cedex 94275, France

Communicated by P.Chambon

The effects of ligand binding and receptor phosphorylation on the interaction of progesterone receptor with specific DNA sequences in the uteroglobin gene were studied by nitrocellulose filter binding and DNase I footprinting. High affinity sites were mapped upstream from the transcription start and in the first intron. They contained a common TGTTCACT sequence. These sites were occupied with similar affinity by the receptor, either in its free state, or complexed with the hormone or an antagonist (RU486); and also by receptor which had been phosphorylated *in vivo* in a hormone-dependent manner. In all cases identical footprints were observed. These experiments led to the following conclusions. (i) The hormone-dependency of receptor binding to DNA or chromatin is observed in intact cells and in crude cellular extracts but not with purified receptor. Thus *in situ*, the unliganded receptor probably interacts with some nuclear component(s) which stabilizes it in a 'non-activated' form (non-chromatin and non-DNA binding form). When isolated, the receptor may undergo activation, even in the absence of the hormone. (ii) Binding by receptor of an antihormone (and possibly receptor phosphorylation) exerts an effect on gene transcription through a mechanism which is different from (and probably follows) receptor interaction with the gene.

Key words: DNase footprinting/hormone effect/progesterone receptor/protein-DNA interaction/uteroglobin gene

Introduction

Steroid hormones bind to intranuclear receptors which in turn regulate transcription of specific genes (Eriksson and Gustafsson, 1983). Recently, the purification of receptors and cloning of hormone-regulated genes has enabled their mechanism of interaction to be studied directly (Payvar *et al.*, 1981, 1983; Govindan *et al.*, 1982; Geisse *et al.*, 1982; Compton *et al.*, 1983; Scheidereit *et al.*, 1983). It was shown that DNA regions exhibiting high affinity towards receptors existed upstream and sometimes within the genes (Payvar *et al.*, 1983; Moore *et al.*, 1985; Slater *et al.*, 1985). In several cases the function of these DNA segments was tested by experiments involving the introduction into recipient cells of genes containing or lacking these sequences (Robins *et al.*, 1982; Chandler *et al.*, 1983; Hynes *et al.*, 1983; Dean *et al.*, 1983; Karin *et al.*, 1984; Renkawitz *et al.*, 1984). The overall mechanism suggested by these studies was relatively simple: the receptor interacted with enhancer-like DNA sequences present near to or inside specific genes. The role of the hormone was to induce an appropriate receptor conformation thereby allowing it to interact with the regulatory regions of DNA (Parker, 1983). However, all these conclusions were based on experiments involving preformed steroid–receptor complexes since no method was available to obtain purified unliganded receptors and thus to study directly the effect of the hormone in these systems.

Moreover, we have recently shown that upon administration of the hormone *in vivo*, the progesterone receptor undergoes a hormone-dependent phosphorylation (Logeat *et al.*, 1985b). The phosphorylated, tightly nuclear-bound species may perhaps be the form of receptor–steroid complex which modulates gene transcription. For this reason it appeared important to analyse its DNA binding properties.

The mechanism of action of steroid antagonists is not presently understood. In most cases these compounds bind to steroid receptors and provoke their activation (tight binding to chromatin or DNA). This has been shown for instance, to be the case for antioestrogens such as tamoxifen (Sutherland and Murphy, 1982; Evans *et al.*, 1982) and for the antiprogestin RU486 (Rauch *et al.*, 1985). To explain why these compounds have antagonistic activity a hypothesis was proposed in which antihormone–receptor complexes bind to DNA non-specifically but do not bind with high affinity to regulatory regions of specific genes (Evans *et al.*, 1982; Rauch *et al.*, 1985).

Using monoclonal antireceptor antibodies we have recently devised a method allowing the purification by immunoaffinity chromatography of active receptor, either complexed to various ligands, or free (Logeat *et al.*, 1985a). We have used this methodology to map exactly the high affinity binding sites for progesterone receptor on and around the uteroglobin gene. We have then studied the effect of hormone, antihormone and receptor phosphorylation on receptor binding to these sites.

Results

Regions of the rabbit uteroglobin gene which bind with high affinity to ligand-free, agonist-bound, antagonist-bound and 'nuclear' progesterone receptor

Initial experiments involved agonist (ORG 2058)–receptor complexes purified by immunoaffinity chromatography from uterine cytosol. [We shall call 'cytosolic' the receptor loosely bound to the nucleus (Perrot-Applanat *et al.*, 1985, 1986) and recovered in the cytosol after homogenization of uteri from progesterone-devoid animals.] The purified complexes were incubated with various ^{32}P-end labeled fragments of DNA encompassing the rabbit uteroglobin gene subcloned in pBR322 or pBR325. After incubation with receptor, the radioactive DNA was filtered onto nitrocellulose and the filters were washed. The DNA fragments which were retained were eluted, electrophoresed on agarose gel and detected by autoradiography. In each case the retention of the fragment of the uteroglobin gene was compared to the retention of a fragment of similar size originating from the plasmid. *Eco*RI fragments of the uteroglobin gene were first studied (Figure 1A). Only the 5-kb fragment corresponding to the first exon, part of the first intron and 5′ flanking regions exhibited

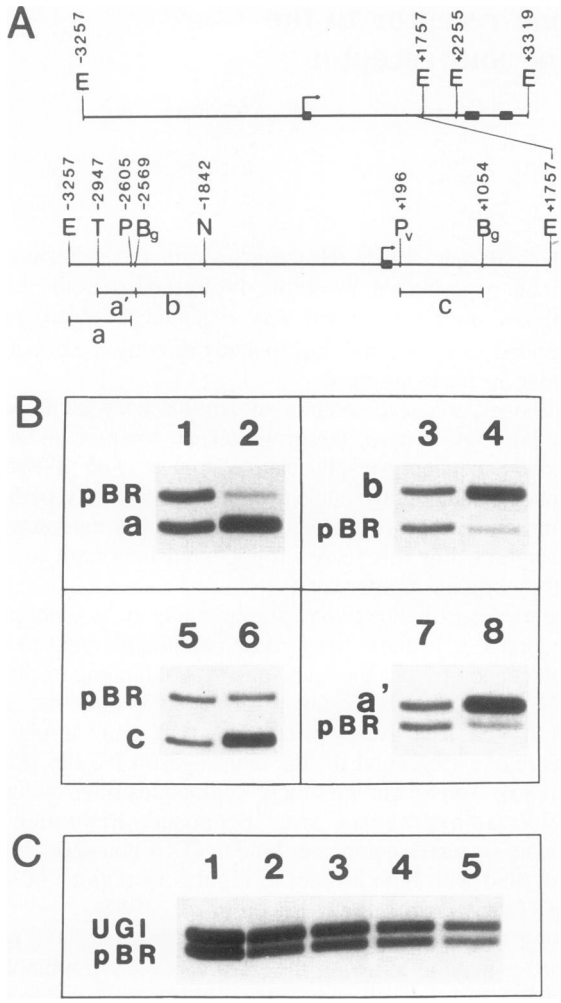

Fig. 1. Filter binding studies of the interaction of progestin–receptor complexes with DNA fragments encompassing the rabbit uteroglobin gene. **(A)** Schematic representation of the DNA in the region of the uteroglobin gene. Restriction sites: *Bgl*II = Bg; *Eco*RI = E; *Nde*I = N; *Pst*I = P; *Pvu*II = Pv; *Taq*I = T. Shaded boxes represent exons. The arrow indicates the site of transcription initiation. **(B)** Fragments of the uteroglobin gene preferentially retained by progestin–receptor complexes. For clarity only the autoradiographic pattern given by the 'specific' DNA fragment and a 'non-specific' (plasmid) DNA fragment of similar size are shown. **Lanes 1, 3, 5** and **7** show the mixture of 'specific' and 'non-specific' (pBR) fragments before filtration. **Lane 2**: filter retention of fragment a (−3256 to −2605). **Lane 4**: filter retention of fragment b (−2568 to −1842). **Lane 6**: filter retention of fragment c (+197 to +1054). **Lane 8**: filter retention of fragment a' (−2946 to −2569). The 'non-specific' pBR322 plasmid DNA fragments were: a 748-bp *Pst*I–*Eco*RI fragment in **lanes 1** and **2**, a 616-bp *Taq*I–*Taq*I fragment in **lanes 3** and **4**, a 1050-bp *Ava*I–*Bam*HI fragment in **lanes 5** and **6**, a 342-bp *Taq*I–*Eco*RI fragment in **lanes 7** and **8**. Concentration of ORG 2058–receptor complexes was 15 pmol/ml. **(C)** Lack of clearcut preferential filter retention of the 'promoter' region of the uteroglobin gene. ^{32}P-labeled 'promoter' DNA (nucleotides −394 to +10) was incubated with ORG 2058–receptor complexes 15 pmol/ml) in presence of increasing concentrations of unlabeled calf thymus DNA. **Lane 1**: DNA fragments before filtration. **Lanes 2–4** and **5**: DNA fragments retained by filters after incubation with 0, 0.5, 1 and 2 μg/ml of calf thymus DNA, respectively. UG1: 'promoter' region of the uteroglobin gene, pBR: 377-bp *Eco*RI–*Bam*HI fragment of pBR322.

preferential binding (not shown). The remaining fragments corresponding to the last two exons, the distal part of the first intron, the second intron, and the 3′ flanking region, were not bound with high affinity (not shown). No preferential binding was observed with nucleotide fragments −425 to +1196 of a non-hormone regulated gene, the rabbit β globin gene (not shown). The 5-kb *Eco*RI fragment was then analysed in detail (Figure 1B). Three regions exhibited clearcut preferential retention. Two were localized far upstream from the transcription initiation site: a fragment extending from nucleotides −3256 to −2605 (further analysis showed that high affinity binding sites were actually present between nucleotides −2946 and −2605) and the adjacent fragment (−2568 to −1842). The third preferentially retained fragment was localized in the first intron (between nucleotides +197 and +1054). The promoter fragment (Bailly *et al.*, 1983) (from nucleotides −394 to +10) was not preferentially retained, even when the calf thymus DNA concentration was increased in order to widen differences between 'specific' and 'non-specific' (vector) DNA binding (Figure 1C).

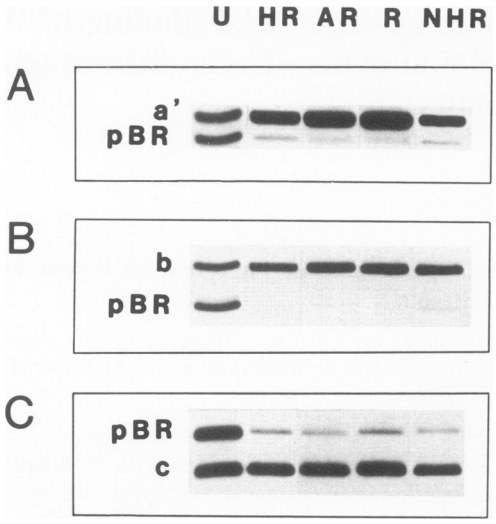

Fig. 2. Regions of the uteroglobin gene which bind with high affinity to ligand-free (R), agonist-bound (HR), antagonist-bound (AR) and 'nuclear' (NHR) progesterone receptor. **(A)** Fragment a′ (nucleotides −2946 to −2569); **(B)** fragment b (nucleotides −2568 to −1842); **(C)** fragment c (nucleotides +197 to +1054). Experimental conditions as in Figure 1. **Lane U** shows the autoradiograms of [^{32}P]DNA fragments not bound by receptor and not filtered onto nitrocellulose; a′, b and c fragments of the uteroglobin gene and the pBR322 fragments of similar size are the same as in Figure 1B. Receptor concentrations: HR (R5020–receptor complexes) = 38 pmol/ml, AR (RU486–receptor complexes) = 28 pmol/ml, R = 32 pmol/ml, NHR ('nuclear' R5020–receptor complexes) = 22 pmol/ml.

These experiments were repeated using ligand-free and antagonist (RU486)-bound immunopurified 'cytosolic' progesterone receptor. In both cases the same three fragments of the uteroglobin gene were preferentially bound by the receptor (Figure 2).

We have recently shown in hormone-treated cells that whilst the receptor binds tightly to chromatin, it undergoes a phosphorylation reaction (Logeat *et al.*, 1985b). Thus, nuclear-bound phosphorylated agonist–receptor complexes may be the active form of receptor. It appeared important to study their interaction with the uteroglobin gene DNA. The potent synthetic agonist R5020 was administered to rabbits and steroid–receptor complexes were purified by immunoaffinity chromatography from nuclear extracts. The 'nuclear' receptor also exhibited high affinity to the same three fragments of the DNA in the region of the uteroglobin gene (Figure 2).

Comparison of affinities of free receptor, receptor–agonist ('cytosolic' and 'nuclear') and receptor–antagonist complexes for specific DNA fragments of the uteroglobin gene

Free, agonist and antagonist-bound receptors have been shown

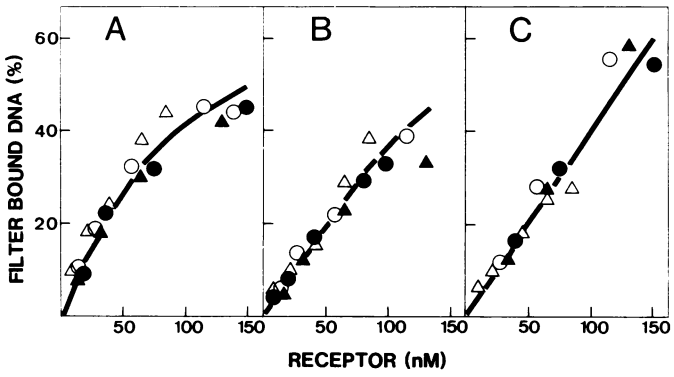

Fig. 3. Comparison of affinities of free receptor, receptor−agonist ('cytosolic' and 'nuclear') and receptor−antagonist complexes for specific DNA fragments of the uteroglobin gene. 'Cytosolic' R5020−receptor complexes (●), 'cytosolic' RU486−receptor complexes (○), free receptor (▲), 'nuclear' phosphorylated R5020−receptor complexes (△) were incubated with fragment a' (nucleotides −2946 to −2569) (**A**), fragment b (nucleotides −2568 to −1842) (**B**) and fragment c (nucleotides +197 to +1054) (**C**). After filtration, gel electrophoresis, autoradiography and scanning, retention of each fragment was compared to the signal given by the non-filtered fragment.

to interact specifically with the same three fragments of the uteroglobin gene. The possibility remained, however, that the hormone or the antiprogestin exerted a more subtle effect by modifying the affinity of receptor for these specific DNA fragments. To test this hypothesis we incubated each radioactively labeled DNA fragment with varying concentrations of unliganded or liganded receptor. After filtration onto nitrocellulose, bound radioactive DNA was eluted, electrophoresed, autoradiographed and the fraction of protein-bound radioactivity was computed. As shown in Figure 3, 'cytosolic' receptor whether free, bound to an agonist or bound to an antagonist interacted with similar affinities with the three specific fragments of the uteroglobin gene.

The same experiment was repeated using 'nuclear' receptor−agonist complexes. Again the affinity for the three specific regions of the uteroglobin gene was found to be identical to that of the 'cytosolic' receptor (Figure 3).

These results were independent of the temperature of incubation of receptor with DNA: similar results were obtained at 0 and 25°C (data not shown).

Comparison of binding sites of free receptor, receptor−agonist ('cytosolic' and 'nuclear') and receptor−antagonist complexes by DNase I footprinting

The aim of these experiments was two-fold. Firstly, it was necessary to define precisely the DNA sequences with which hormone−receptor complexes interacted inside the three DNA fragments shown to specifically bind the receptor. Secondly, it was necessary to compare the footprints obtained with receptor under its various forms. Indeed, it was possible that due to the effect of the hormone, of the antihormone, or after phosphorylation the receptor interacted with some sites but not with others. Moreover, even if it was interacting with the same sites it could yield different nuclease footprints due to differences in the affinity ('tightness') of binding or to changes in its oligomeric composition or conformation.

Figures 4−6, show examples of footprint experiments (Galas and Schmitz, 1978). Figure 7A describes in detail all the observations made during the analysis of both DNA strands and with all three DNA fragments which specifically interacted with the receptor. The extent of protection by receptor against DNase I

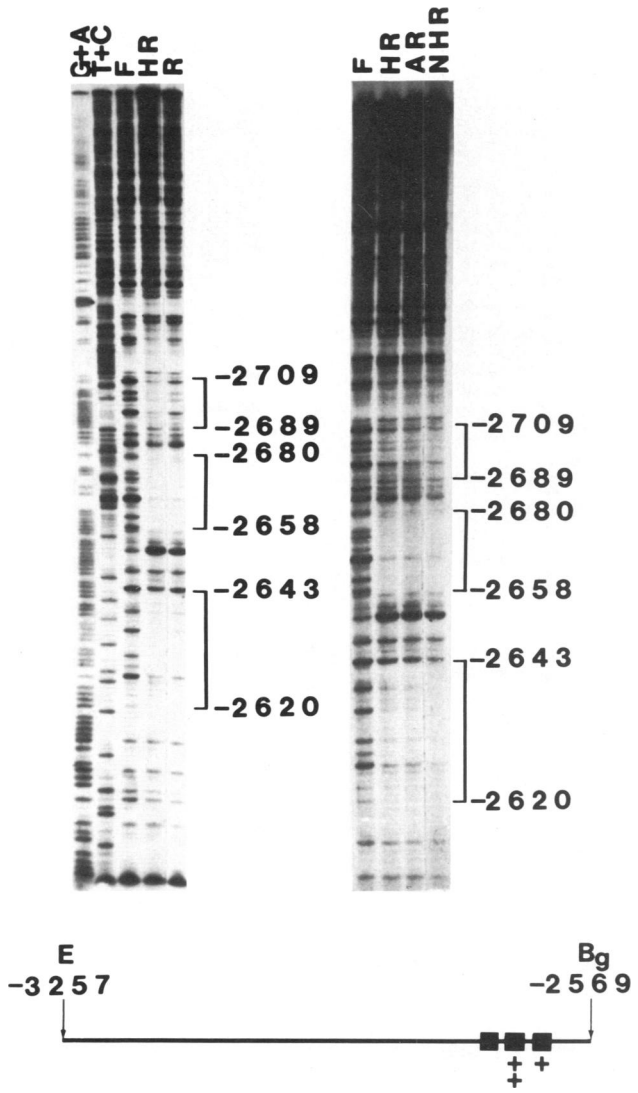

Fig. 4. DNase I footprints. Receptor interaction with the *Eco*RI (E)−*Bgl*II (Bg) fragment (nucleotides −3256 to −2569). G+A and T+C: Maxam and Gilbert sequence ladder. F: free DNA (in the absence of receptor). The DNA was incubated with the following steroid−receptor complexes: HR, 'cytosolic' hormone (R5020)−receptor complexes; AR, 'cytosolic' antihormone (RU486)−receptor complexes; and NHR, 'nuclear' hormone (R5020)−receptor complexes, or with free receptor (R). The DNA fragment (non-coding strand) was 5'-end labeled at the *Bgl*II site. The three protected sites are schematized in the lower part of the figure (their exact boundaries are given in the margin of the autoradiograms). Intensity of protection by receptor has been evaluated semi-quantitatively: $\overset{+}{+}$ = site very strongly protected, + = site strongly protected, no symbol = average protection.

digestion, under comparable experimental conditions allowed the binding sites to be classified. Similar degrees of protection observed on both DNA strands confirmed this classification. Two sites exhibited a very high affinity: one was localized between nucleotides −2680 and −2655, the other between nucleotides −2396 and −2376. Three other sites showed a somewhat lower affinity: one between nucleotides −2643 and −2620 and the others in the first intron between nucleotides +297 and +316 and between nucleotides +968 and +1005. Two other clearcut sites, but of somewhat lower affinity, were also observed (see Figures 4 and 5). Enhanced cuts often arose at the boundaries of these sites and various minor modifications were also seen in their proximity (see detail in Figure 7A). For these reasons the limits of the binding sites were sometimes difficult to define.

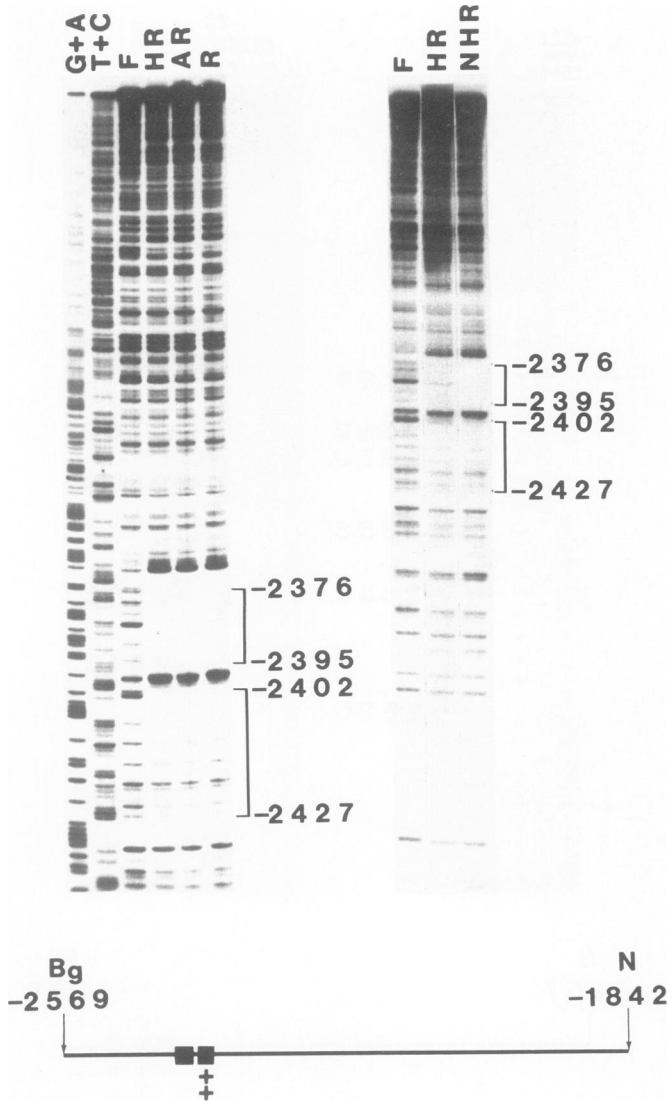

Fig. 5. DNase I footprints. Receptor interaction with the *Bgl*II (Bg)– NdeI (N) fragment (nucleotides −2568 to −1842). For explanation of symbols see Figure 4. DNA was 5′-labeled at the *Bgl*II site (coding strand).

Consequently, we took into consideration regions of contiguous nucleotides protected by receptor.

Experiments using either free receptor, or receptor complexed to agonist or antagonist or 'nuclear' receptor yielded exactly the same footprinting patterns (see examples in Figures 4−6).

Comparison of the different regions protected by the receptor from DNase I digestion showed that they had a uniform size of 23 ± 3 bp. Only one of them was longer (38 bp). In this short stretch of nucleotides a remarkably conserved sequence of 8 bp was observed. TGTTCACT was present in three of the sites, and practically identical sequences were observed in the four other sites (Figure 7B). The longest site (nucleotides +968 to +1005) contained two slightly modified copies of this sequence.

Discussion

The mechanism of interaction between steroid receptors and hormone-regulated genes have lately been the subject of great interest not only because their understanding is important for the elucidation of the mechanism of hormone action, but also because they are one of the few available models in eukaryotes for the analysis of the regulation of gene transcription by defined proteins. This model involves enhancer-like DNA regions which interact with a specific protein (the receptor) when the latter has bound a small regulatory molecule (the steroid). Most of our knowledge in this field has come from the study of glucocorticoid-regulated genes; the mouse mammary tumor virus gene (Pfahl, 1982; Scheidereit *et al.*, 1983; Payvar *et al.*, 1983), the human metallothionein-II_A gene (Karin *et al.*, 1984), the chicken lysozyme gene (Renkawitz *et al.*, 1984), the rabbit uteroglobin gene (Cato *et al.*, 1984), the human growth hormone gene (Moore *et al.*, 1985). Some information is also available in the case of progesterone-regulated genes for chick egg-white proteins (Mulvihill *et al.*, 1982; Compton *et al.*, 1983; Chambon *et al.*, 1984; von der Ahe *et al.*, 1985). In none of these systems has it yet been possible to analyse *in vitro* the role of the hormone or of an antagonist.

We show in the present study that the progesterone receptor binds with high affinity to several DNA sites in the region of the uteroglobin gene and protects them from DNase I digestion. These high affinity binding sites are concentrated in the 5′ flanking region of the gene and in the first intron. For several other hormone-regulated genes, such high affinity binding sites have been shown to be functionally relevant by transformation of recipient cells with truncated or hybrid genes and testing for hormonal stimulation. For technical reasons, similar studies have not been possible in the case of the uteroglobin gene (Cato *et al.*, 1984). However, one of the fragments studied here (nucleotides −2711 to −2621) has been shown to bind glucocorticoid receptor at a site corresponding to the consensus sequence of the glucocorticoid-regulatory elements (Cato *et al.*, 1984). Moreover, it has also been shown in genes regulated by both glucocorticoid and progesterone (e.g. the chicken lysozyme gene), that the regulatory DNA regions for both hormones are overlapping (Renkawitz *et al.*, 1984; von der Ahe *et al.*, 1985). Thus, at least for one of the uteroglobin gene fragments which binds progesterone receptor with high affinity, there is strong indirect evidence that it may actually be a physiologically relevant regulatory element.

The existence of glucocorticoid regulatory elements in both the 5′ flanking region and inside the gene have been observed in the cases of mouse mammary tumour virus (Payvar *et al.*, 1981, 1983; Geisse *et al.*, 1982) and human growth hormone (Moore *et al.*, 1985; Slater *et al.*, 1985). In the latter gene, one of the regulatory elements was also localized inside the first intron.

The common eight nucleotides motif (TGTTCACT) present in the different sites binding progesterone receptor in the uteroglobin gene resembles the consensus sequence (TGTTCT) of DNA sites which interact with the glucocorticoid receptor (Payvar *et al.*, 1983; Scheidereit and Beato, 1984). This resemblance may explain in part the overlap of the binding sites for both receptors.

Purified receptor binds to the same DNA regions of the uteroglobin gene with similar affinity, whether complexed with an agonist, an antagonist or free. This is in contrast to the situation prevailing in the intact cell where, in the absence of hormone, the receptor is localized in the nucleus but only loosely bound since homogenization even at low ionic strength is sufficient to solubilize it. Under the influence of the hormone, the receptor becomes tightly bound to the chromatin and can only be extracted by buffers of high ionic strength (Eriksson and Gustafsson, 1983). Similar findings were obtained with crude cellular extracts:

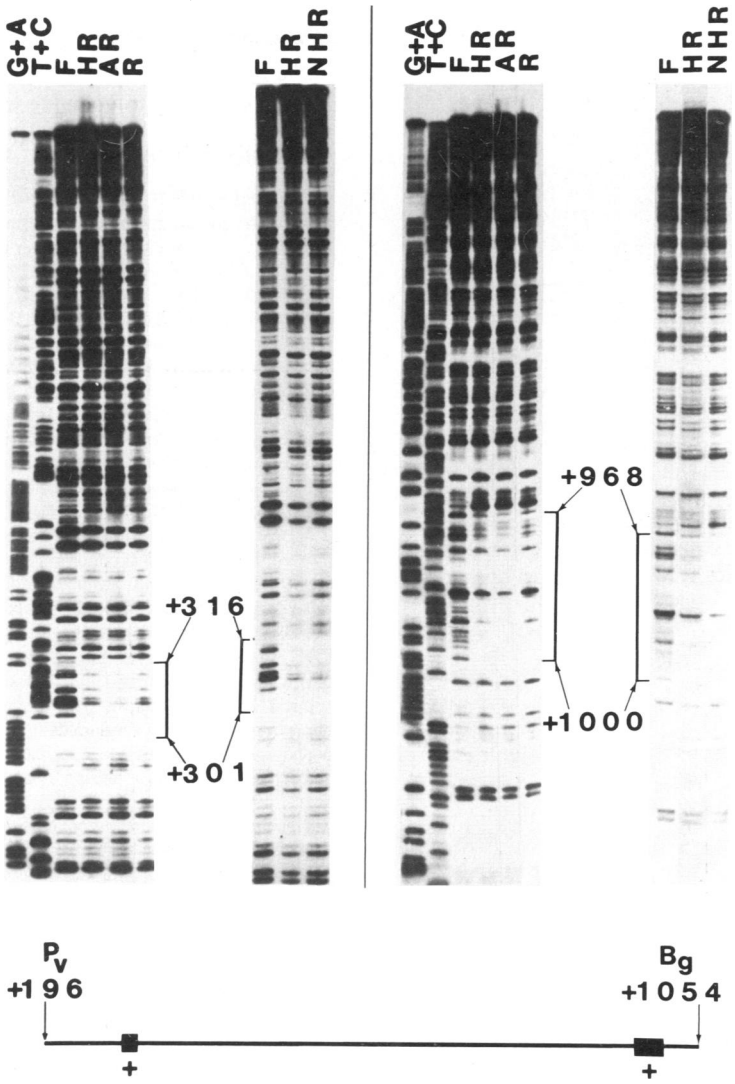

Fig. 6. DNase I footprints. Receptor interaction with the *Pvu*II (Pv)−*Bgl*II (Bg) fragment (nucleotides +197 to +1054). For explanation of symbols see Figure 4. In the two panels on the left DNA was labeled at the *Pvu*II site, in the two panels on the right at the *Bgl*II site. In both cases 5′ labeling was used.

ligand-free receptor does not bind to nuclei or DNA. Thus the effect of the hormone on receptor binding to nuclei and DNA, which is observed in whole cells or crude cellular extracts, cannot be reproduced with purified receptor. The latter has lost a component necessary for the hormone to exert its role. It is probable that in the nucleus of an intact cell and in the absence of hormone the receptor interacts with a factor which stabilizes it in the 'non-activated' form. In the presence of the hormone it becomes activated (binding tightly to DNA or chromatin). When purified (i.e. in the absence of this factor) the receptor may adopt spontaneously the activated conformation, even without hormone. This 'aporeceptor activation inhibitor' is probably a nuclear protein. Identification and isolation of this nuclear component will be necessary to understand the molecular changes which accompany hormone binding to receptor. The simple allosteric model which explains the function of some prokaryotic proteins, such as the effect of cyclic AMP on the interaction of its binding protein (CRP) with specific genes (Takeda *et al.*, 1983), thus does not seem to apply to the progesterone receptor.

A 90-kd heat-shock protein has been described which in the presence of molybdate interacts with chick progesterone receptor (Sanchez *et al.*, 1985; Catelli *et al.*, 1985), as well as other steroid receptors thereby preventing their activation. This protein thus has some functional properties expected of the aporeceptor activation inhibitor. However, it is still not clear if the interaction between this heat-shock protein and receptor takes place in the intact cell or if it is an artefact of tissue homogenization and addition of molybdate. Moreover, it has been shown by immunocytochemistry that the 90-kd protein is essentially cytoplasmic whereas the progesterone receptor is intranuclear even in the absence of hormone. Purification in the presence of molybdate yields non-activated (non-DNA binding) receptor (A.Bailly, unpublished observation).

Another change which takes place in target cells upon hormone administration is receptor phosphorylation (Logeat *et al.*, 1985b; Pike and Sleator, 1985). This reaction does not change the interaction of receptor with the uteroglobin gene. Thus receptor phosphorylation, if it plays a role in the regulation of gene expression, acts at step(s) following receptor binding to the gene.

We have previously studied by a filter retention method the binding to the uteroglobin gene of 30−50% pure receptor preparations obtained through a purification scheme relying mainly on DNA−cellulose chromatography (Bailly *et al.*, 1983). With these preparations we observed binding to the same regions of the uteroglobin gene as described above, but we also found binding to a fragment extending from nucleotides −394 to +10.

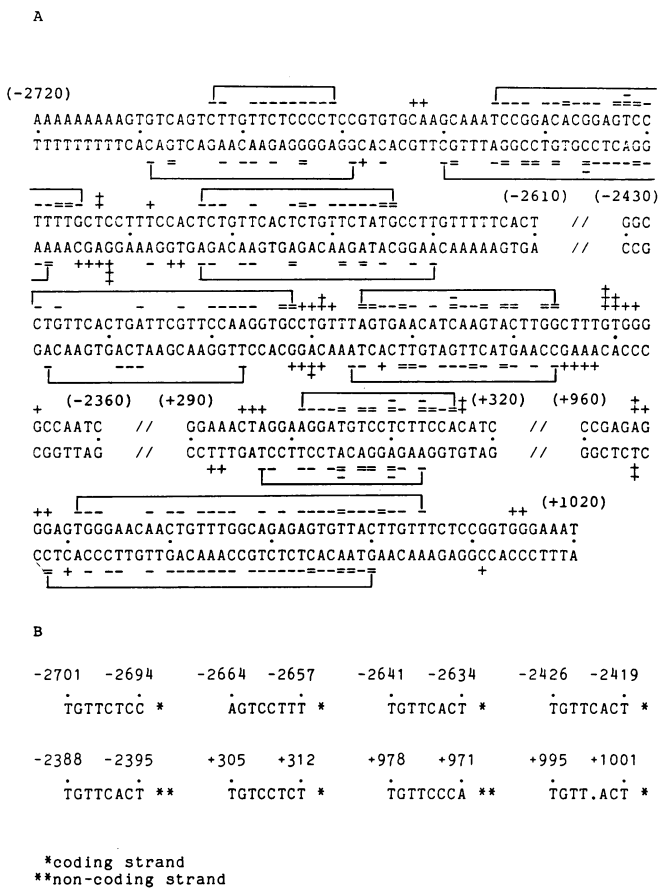

Fig. 7. (**A**) DNase I digestion. Effect of progesterone receptor binding to the uteroglobin gene. The three DNA fragments to which progesterone receptor bound with high affinity were completely studied and on both strands by the DNase I digestion method. The modifications observed on each DNA strand, upon binding of receptor are reported. (−) indicates a decrease of cut by DNase when compared to control (absence of receptor); (+) indicates an enhanced cut. The intensity of the modifications was approximately determined by visual inspection and is reported using a scale of one, two or three + or − signs. Brackets indicate the positions of the binding sites. (**B**) Comparison of the nucleotide sequences of the binding sites for the progesterone receptor. Only the sequence common to all the sites is shown. Positions of the first and last nucleotides are indicated.

High affinity binding to this fragment was not detected with immunopurified receptor. It is thus possible that this interaction was due to a DNA binding protein which copurified with receptor. We are presently studying the possible biological significance of this observation.

Steroid antagonists fall into two categories when considering DNA or chromatin binding of receptors. In a few cases, they bind to receptors but do not provoke their tight binding to DNA or chromatin (Bourgeois *et al.*, 1984). Competition for the steroid binding site and lack of activation may thus explain the antihormonal activity. More difficult to understand is the mechanism of action of the majority of these compounds which activate receptors and make them bind to DNA or chromatin. This is the case for oestrogen receptor upon binding of tamoxifen (or its metabolites) (Evans *et al.*, 1982) and of progesterone receptor upon RU486 binding (Rauch *et al.*, 1985). To explain their antagonistic effect it has been proposed that these antihormones induce a receptor conformation exhibiting affinity towards random DNA sequences but a decreased affinity for the specific regulatory regions of hormone-regulated genes. In the case of the uteroglobin gene and RU486 receptor complexes this explanation does not hold. The mechanism of the antagonistic effect will have to be searched for either at the level of receptor phosphorylation and/or in the reactions which follow receptor binding to specific regions of genes leading to changes in their rate of transcription.

Materials and methods

Purification of rabbit uterine progesterone receptor

Prepubertal rabbits primed with oestrogen were used (Logeat *et al.*, 1985a). 'Cytosolic' receptor either free or after incubation with a ligand (0.1 μM, specific activity 2 Ci/mmol) was purified by immunoaffinity chromatography (Logeat *et al.*, 1985a). Two agonists were used: either [^{3}H]ORG 2058 [16α-ethyl-21-hydroxy-19-norpregna-4-en-3,20-dione] (Amersham) or [^{3}H]R5020 [17,21-dimethyl-19-norpregna-4,9-dien-3,20-dione] (New England Nuclear), the antagonist was: [^{3}H]RU486 [17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(1-propynyl)-estra-4,9-dien-3-one] (Roussel-Uclaf). When [^{3}H]RU486 was employed, 5 μM unlabelled dexamethasone was added to prevent binding to glucocorticoid receptor. In non-hormone incubated receptor preparations endogenous progesterone was measured by radioimmunoassay and shown to be present at a concentration of <0.005 molecule/molecule of receptor.

The specific activity of the receptor was about 65% of the theoretical maximum (Logeat *et al.*, 1985a). Silver or Coomassie Blue staining of polyacrylamide gels showed that the 110 000 dalton receptor and the proteolytic fragments (mainly the 79 000 dalton band) accounted for at least 90% of protein.

To prepare 'nuclear' steroid−receptor complexes, rabbits were injected with R5020, nuclei prepared, extracted by high salt and the extract was diluted to lower ionic strength as described (Logeat *et al.*, 1985b). Immunoaffinity chromatography purification was performed under the same conditions as for 'cytosolic' receptor (Logeat *et al.*, 1985a,b). The elution conditions did not modify receptor phosphorylation. Polyacrylamide gel electrophoresis in presence of sodium dodecylsulfate showed the decrease in electrophoretic mobility which is characteristic of receptor phosphorylated after administration of hormone (Logeat *et al.*, 1985b).

To all receptor preparations bovine serum albumin (BRL nuclease free, 3 mg/ml) was added and dialysis (6 h at 4°C) was performed against 20 mM Tris−HCl pH 7 buffer containing dithiothreitol, 2 mM, and unlabelled steroid, 0.1 μM (except for ligand-free receptor). Receptor was either used immediately or kept frozen in liquid nitrogen. Since there were slight differences in the DNA-binding properties between batches of purified receptor we always compared receptors purified in the same experiment (agonist versus antagonist-bound receptor, ligand-free versus agonist-bound receptor, etc.).

To test the possibility of copurification of contaminating DNA binding proteins with receptor we performed a 'mock purification' using an immunomatrix synthesized with a non-receptor related monoclonal antibody [this antibody, of the same Ig_{2a} class as the antireceptor antibody, is described in Logeat *et al.* (1985a)]. The eluate of this column was shown to be devoid of any DNA binding activity. Moreover it was possible to remove the DNA binding activity from the receptor preparation with antireceptor monoclonal antibody (a non-related antibody had no effect).

Assay of progesterone receptor

The concentration of agonist or antagonist−receptor complexes purified from cytosol was determined by using radioactive steroid and measuring the concentration of steroid−receptor complexes eluted from the immunomatrix (Logeat *et al.*, 1985a). This method was not applicable, however, to ligand-free receptor, or to the nuclear receptor, since it was not possible to administer sufficient amounts of radioactive hormone to rabbits. Thus we devised an immunoblot method to measure receptor concentration. Dots (2.5−100 pmol receptor/ml; 2 μl) were applied to nitrocellulose. After drying they were incubated with antireceptor Mi60 monoclonal antibody, an anti-mouse immunoglobulin antibody and radioactive protein A as previously described for Western blots (Logeat *et al.*, 1985a). After washing and autoradiography, the radioactivity in the dots was measured by scanning. The assay was linear for receptor amounts varying between 2.5 and 40 pmol/ml.

Uteroglobin gene DNA

The two recombinant λ phages carrying the uteroglobin gene inserts have been described previously (Atger *et al.*, 1981). Corresponding sequences have been established (Bailly *et al.*, 1983; Suske *et al.*, 1983; Cato *et al.*, 1984) and the nucleotide numbering corrected due to the omission of 4 bp in a previously published sequence (Cato *et al.*, 1984). All *Eco*RI fragments were subcloned in pBR325. *Eco*RI−*Bam*HI and *Bam*HI fragments were subcloned in pBR322.

After digestion with the appropriate restriction enzyme the DNA was 5′ end-labeled (Maniatis *et al.*, 1982).

Study of receptor−DNA interactions by filter-binding

Receptor (concentrations are stated in figure legends) was incubated for 2 h at

0°C with ^{32}P-labelled DNA fragments (0.04–0.33 pmol/ml) in 120 μl of Tris 20 mM HCl pH 7.0 buffer containing dithiothreitol, 2 mM; EDTA, 0.3 mM; NaCl, 150 mM; calf thymus DNA, 1 μg/ml; nuclease-free bovine serum albumin (1 or 2 mg/ml). If steroid–receptor complexes were used, unlabeled steroid (0.1 μM) was added.

After filtration at 4°C onto nitrocellulose (Bailly *et al.*, 1983), each filter was washed twice with 0.3 ml of Tris 10 mM, NaCl 50 mM HCl pH 7.4 buffer. Radioactive DNA bound to the filter was counted and eluted as described (Payvar *et al.*, 1983). In some experiments several filters were pooled. After ethanol-precipitation DNA was electrophoresed on 1 or 2% agarose gels. Non-filtered, ^{32}P-labeled DNA fragments, at a concentration allowing comparison with receptor-bound DNA, were also electrophoresed.

Study of receptor DNA interaction by DNase I footprinting

A DNA fragment ^{32}P-labeled (0.18–1.16 nM) at one 5′ or 3′ end (Maniatis *et al.*, 1982) was incubated for 90 min at 4°C in the presence or in the absence of receptor (55–110 pmol/ml) in 60 μl of the buffer described above for filter binding studies (with the following modifications: EDTA was omitted, NaCl lowered to 50 mM, $MgCl_2$ 0.2 mM and $CaCl_2$ 0.2 mM were added). DNA was then digested for 30 min at 4°C with DNase I (DPFF, Worthington) (2 μl of a solution of 6 μg/ml). Digestion was stopped by addition of 20 mM EDTA and 2 μg of calf thymus DNA. After phenol extraction and ethanol precipitation, DNA fragments were analysed on 6% polyacrylamide gels calibrated with a sequence ladder obtained by the method of Maxam and Gilbert (1980).

Acknowledgements

We are grateful to Dr C.Mugnier (Centre Inter-universitaire de Traitement de l'Information no.2 Paris) for computer studies of the DNA sequences and comparisons with sequence libraries. We thank Dr D.Philibert (Roussel-Uclaf, Romainville) for the gift of RU486. The manuscript was typed by N.Malpoint and M.Messina. This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM), the Unité d'Enseignement et de Recherche Kremlin-Bicêtre, the Association pour la Recherche sur le Cancer (ARC) and the Fondation pour la Recherche Médicale Française.

References

Atger,M., Atger,P., Tiollais,P. and Milgrom,E. (1981) *J. Biol. Chem.*, **256**, 5970–5972.

Bailly,A., Atger,M., Atger,P., Cerbon,M.A., Alizon,M., Vu Hai,M.T., Logeat,F. and Milgrom,E. (1983) *J. Biol. Chem.*, **258**, 10384–10389.

Bourgeois,S., Pfahl,M. and Baulieu,E.E. (1984) *EMBO J.*, **3**, 751–755.

Catelli,M.G., Binart,N., Jung-Testas,I., Renoir,J.M., Baulieu,E.E., Feramisco,J.R. and Welch,W.J. (1985) *EMBO J.*, **4**, 3131–3135.

Cato,A.C.B., Geisse,S., Wenz,M., Westphal,H.M. and Beato,M. (1984) *EMBO J.*, **3**, 2771–2778.

Chambon,P., Dierich,A., Gaub,M.P., Jakowlev,S, Jongstra,J., Krust,A., LePennec,J.P., Oudet,P. and Reudelhuber,T. (1984) *Recent Prog. Horm. Res.*, **40**, 1–39

Chandler,V.L., Maler,B.A. and Yamamoto,K.R. (1983) *Cell*, **33**, 489–499.

Compton,J.G., Schrader,W.T. and O'Malley,B.W. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 16–20.

Dean,D.C., Knoll,B.J., Riser,M.E. and O'Malley,B.W. (1983) *Nature*, **305**, 551–554.

Eriksson,H. and Gustafsson,J.A. (eds) (1983) *Steroid Hormone Receptors: Structure and Function. Nobel Symposium no. 57.* Elsevier Science Publishers, Amsterdam.

Evans,E., Baskevitch,P.P. and Rochefort,H. (1982) *Eur. J. Biochem.*, **128**, 185–191.

Galas,D.J. and Schmitz,A. (1978) *Nucleic Acids Res.*, **5**, 3157–3170.

Geisse,S., Scheidereit,C., Westphal,H.M., Hynes,N.E., Groner,B. and Beato,M. (1982) *EMBO J.*, **1**, 1613–1619.

Govindan,M.V., Spiess,E. and Majors,J. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 5157–5161.

Hynes,N., van Ooyen,A.J.J., Kennedy,N., Herrlich,P., Ponta,H. and Groner,B. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3637–3641.

Karin,M., Haslinger,A., Holtgreve,H., Richards,R.I., Krauter,P., Westphal,H.M. and Beato,M. (1984) *Nature*, **308**, 513–519.

Logeat,F., Pamphile,R., Loosfelt,H., Jolivet,A., Fournier,A. and Milgrom,E. (1985a) *Biochemistry*, **24**, 1029–1035.

Logeat,F., Le Cunff,M., Pamphile,R. and Milgrom,E. (1985b) *Biochem. Biophys. Res. Commun.*, **131**, 421–427.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York.

Maxam,A.M. and Gilbert,W.C. (1980) *Methods Enzymol.*, **65**, 499–560.

Moore,D.D., Marks,A.R., Buckley,D.I., Kapler,G., Payvar,F. and Goodman,H.M. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 699–702.

Mulvihill,E.R., LePennec,J.P. and Chambon,P. (1982) *Cell*, **28**, 621–632.

Parker,M. (1983) *Nature*, **304**, 687–688.

Payvar,F., Wrange,O., Carlstedt-Duke,J., Okret,S., Gustafsson,J.A. and Yamamoto,K.R. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 6628–6632.

Payvar,F., DeFranco,D., Firestone,G.L., Edgar,B., Wrange,O., Okret,S., Gustafsson,J.A. and Yamamoto,K.R. (1983) *Cell*, **35**, 381–392.

Perrot-Applanat,M., Logeat,F., Groyer-Picard,M.T. and Milgrom,E. (1985) *Endocrinology*, **116**, 1473–1484.

Perrot-Applanat,M,. Groyer-Picard,M.T., Logeat,F. and Milgrom,E. (1986) *J. Cell Biol.*, **102**, 1191–1199.

Pfahl,M. (1982) *Cell*, **31**, 475–482.

Pike,J.W. and Sleator,N.M. (1985) *Biochem. Biophys. Res. Commun.*, **131**, 378–385.

Rauch,M., Loosfelt,H., Philibert,D. and Milgrom,E. (1985) *Eur. J. Biochem.*, **148**, 213–218.

Renkawitz,R., Schutz,G., von der Ahe,D. and Beato,M. (1984) *Cell*, **37**, 503–510.

Robins,D.M., Paek,I., Seeburg,P.H. and Axel,R. (1982) *Cell*, **29**, 623–631.

Sanchez,E.R., Toft,D.O., Schlesinger,M.J. and Pratt,W.B. (1985) *J. Biol. Chem.*, **260**, 12398–12401.

Scheidereit,C. and Beato,M. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 3029–3033.

Scheidereit,C., Geisse,S., Westphal,H.M. and Beato,M. (1983) *Nature*, **304**, 749–752.

Slater,E.P., Rabenau,O., Karin,M., Baxter,J.D. and Beato,M. (1985) *Mol. Cell. Biol.*, **5**, 2984–2992.

Suske,G., Wenz,M., Cato,A.C.B. and Beato,M. (1983) *Nucleic Acids Res.*, **11**, 2257–2271.

Sutherland,R.L. and Murphy,L.C. (1982) *Mol. Cell. Endocrinol.*, **25**, 5–23.

Takeda,Y., Ohlendorf,D.H., Anderson,W.F. and Matthews,B.W. (1983) *Science*, **221**, 1020–1026.

von der Ahe,D., Janich,S., Scheidereit,C., Renkawitz,R., Schutz,G. and Beato,M. (1985) *Nature*, **313**, 706–709.

Received on 30 July 1986; revised on 8 September 1986